clinical chemistry

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Reprinted from February 1993

CLIN. CHEM. 39/2, 244–247 (1993)

Nonradioactive, Colorimetric Microplate Hybridization Assay for Detecting Amplified Human Immunodeficiency Virus DNA

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A nonradioactive, colorimetric microplate hybridization procedure was used to assay human immunodeficiency virus (HIV) DNA, amplified by the polymerase chain reaction (PCR). Under the PCR conditions used, four proviral copies per 150 000 cells were detected by amplifying a series of DNA mixtures that contained various copy numbers of HIV. Assays of PCR-amplified DNA from peripheral blood mononuclear cells of seronegative individuals yielded negative results (104 of 104), whereas samples from seropositive individuals yielded >99% positive results (141 of 142). Similar results were obtained in a chemiluminescent assay with an acridinium ester-labeled probe and in a solution hybridization assay in which a ^{32}P-labeled probe was used.

Indexing Terms: *polymerase chain reaction · mononuclear blood cells*

The polymerase chain reaction (PCR) may be used to amplify very small quantities of nucleic acid up to several million times (*1*).[3] Nucleic acids of infectious agents, such as human immunodeficiency virus (HIV), that are frequently not detectable by conventional molecular biology methods can be amplified to detectable amounts by PCR. The amplified HIV DNA is identified by means of DNA probes labeled by various isotopic (*2, 3*) and nonisotopic methods (*4–7*). Tests with ^{32}P-labeled DNA probes usually require 3–18 h and are complicated by the isotope's short half-life and the potential biohazards of the radiolabeled detector probes. Here we report a colorimetric, nonradioactive microplate hybridization assay for detecting amplified HIV DNA.

Materials and Methods

Patients, Cells, and PCR

Peripheral blood mononuclear cell (PBMC) samples from 142 individuals who acquired HIV-1 infection through homosexual activity or through transfusion of blood or blood products were used for this study. These samples were first examined by PCR amplification of the PBMC DNA and by acridinium ester (AE)-labeled probe hybridization assays (*6*) of the amplified DNA. PBMC samples from 104 HIV-seronegative individuals with no known high-risk behavior were included as negative controls. PBMCs were isolated from whole blood by using Ficoll-Hypaque and were frozen in 100 mL/L dimethylsulfoxide at −70 °C until testing. DNA processing from the PBMC samples with a lysis buffer containing detergents and proteinase K and the conditions for PCR were described previously (*8*). Frozen cells were washed with cold phosphate-buffered saline and lysed with PCR lysis buffer containing, per liter, 50 mmol of KCl; 10 mmol of Tris · HCl, pH 8.3; 2.5 mmol of $MgCl_2$; 4.5 mL each of NP40 and Tween 20; and freshly added proteinase K (6 mL of 10 g/L proteinase K per liter of lysis buffer). The concentration of cells was adjusted to ~6 × 10^6 cells/mL of lysis buffer. The mixture was incubated at 56 °C for 1 h and then at 95 °C for 10 min. About 1 μg of DNA from each sample, representing DNA from 150 000 PBMCs, was subjected to PCR with the SK38/39 primer pair (ATAATCCACCTATCCCAG AGGAGAAAT/TTTGGTCCTTGTCTTATGTCCAGAATGC) specific for a sequence of the p24 *gag* gene (*2*). Amplification was carried out for 35 cycles in a DNA thermal cycler (Perkin Elmer/Cetus, Norwalk, CT) as previously described (*8*).

HIV DNA Standards and Cloned DNA

DNA samples containing 0, 2, 4, 8, 16, 32, 64, 125, 250, 500, or 1000 copies of HIV-1 proviral DNA were prepared by mixing DNA extracted from 8E5 cells (*9*), which carry a single HIV-1 provirus per cell, and a constant amount of HIV-negative DNA derived from a seronegative individual. The 8E5 DNA samples and the HIV-negative DNA samples were prepared similarly to DNA samples from seropositive individuals.

Detection of Amplified DNA

Colorimetric microplate assay. The assay for amplified HIV DNA sequences (Figure 1) was adapted from the colorimetric microplate hybridization assay described by Cook et al. (*10*). All incubations were at room temperature. PCR reaction products (5 μL) or their dilutions were denatured with 15 μL of 0.5 mol/L NaOH for 15 min at room temperature and then hybridized to a capture oligonucleotide (5′-TGGTAGGGCTATACAT-

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[3] Nonstandard abbreviations: PCR, polymerase chain reaction; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; AE, acridinium ester; HRP, horseradish peroxidase; SSC, standard saline citrate (1× SSC is 8.765 g of NaCl and 4.41 g of sodium citrate per liter, pH 7.0); and RLU, relative light units.
Received December 3, 1991; accepted September 11, 1992.

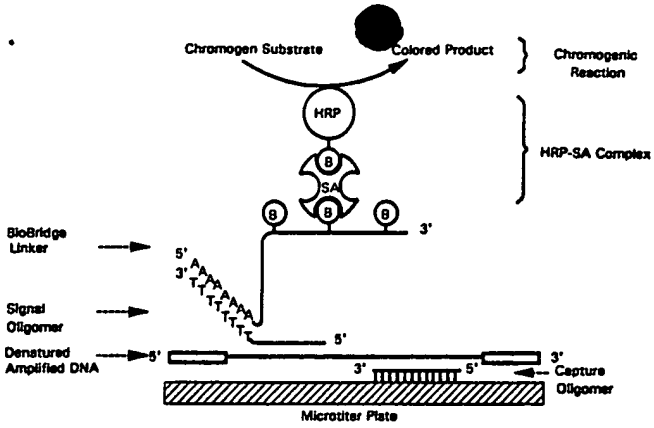

Fig. 1. Detection of PCR-amplified HIV DNA with the microplate hybridization assay

The box at the 5′ end of the amplified product represents the SK38 sequence and the box at the 3′ end represents the sequence complementary to that of SK39. *B*, biotin; *SA*, streptavidin; *HRP*, horseradish peroxidase

TCTTACTATTTTATTTAATCCCA GGATTATCCAT-3′) that had been affixed to microplate wells (*11*). Hybridization in microwells was for 90 min with shaking in 100 μL of hybridization mixture containing, per liter, 250 g of dextran sulfate, 330 mL of formamide, 120 mmol of HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.0], and 150 mmol of NaCl. Wells were emptied by inversion and blotting. Captured HIV sequences were then hybridized for 15 min in 100 μL of hybridization mixture containing poly T-tailed signal oligonucleotide, KS 38, whose sequence is complementary to primer SK 38. The resulting hybrid was washed five times with 0.2× standard saline citrate (SSC) containing 1 mL of Triton X-100 per liter. Biotin-labeled poly dA (BioBridge® Labeling Molecule; Enzo Diagnostics, Inc., Syosset, NY) was hybridized to the captured poly T-tailed signal oligonucleotide (Figure 1) in 1.2× SSC containing 1 mL of Triton X-100. The wells were emptied, 40 μL of streptavidin–biotinylated horseradish peroxidase (HRP) complex (DETEK® Hrp; Enzo Diagnostics) was added to each well, and the plate was incubated with shaking for 15 min. After the wells were washed, HRP activity was determined by adding 100 μL of substrate/chromogen mixture (0.5 g/L tetramethylbenzidine in citrate phosphate buffer, pH 5.3, containing H_2O_2, 0.025 mL/L). After 15 min, the reaction was stopped with 100 μL of 1 mol/L H_2SO_4. The blue color generated in positively reacting wells turns to yellow on H_2SO_4 addition. Results were quantified with a microplate reader (Model 345 ATC; SLT Lab Instruments, Research Triangle Park, NC) at 450 nm. Color was stable for ≥30 min after termination of the reaction.

^{32}P-labeled DNA probe assay. One-tenth volume (10 μL) of the amplified product was assayed by using a ^{32}P-labeled SK19 probe (ATCCTGGGATTAAAT-AAAATAGTAAGAATGTATAGCCCTAC) as described (*2, 6*). The NaCl concentration in the reaction volume (15 μL) was 150 mmol/L. The amplified DNA and the DNA probe were mixed and denatured at 95 °C for 3 min, then incubated at 56 °C for 30 min. After hybridization, 7 μL of restriction enzyme reagent containing, per liter, 10 mmol of $MgCl_2$, 30 mmol of Tris · HCl (pH 7.6), and 0.5 U of *Bst* N-I (Bethesda Research Laboratories, Gaithersburg, MD) was added and incubated at 56 °C for 30 min and then at 95 °C for 3 min. After digestion, 5 μL of loading buffer (*6*) was added and 10 μL of the mixture was loaded on a 20% polyacrylamid gel. After electrophoresis, the gel was exposed to a Kodak XOmat film at −70 °C for 120 min.

AE-labeled probe assay. Amplified product (25 μL) was subjected to the AE-labeled probe assay (*6*). In this procedure the amplified DNA was denatured at 95 °C for 5 min and then the DNA probe, a 50-μL mixture of th *gag*-1 and *gag*-2 sequences, was added. The mixture was hybridized at 60 °C for 30 min and the hybridization reaction was terminated quickly by cooling the mixture in ice water for 2 min. Unhybridized probe was inactivated by adding 300 μL of hydrolysis buffer at 60 °C for 10 min. Hybridized probe was quantified in a luminometer (Leader I; GenProbe, San Diego, CA). Photon emission was expressed in relative light units (RLU).

Results

Microplate Hybridization Assay

The microplate hybridization assay (Figure 1) detects DNA sequences according to an enzyme immunoassay format. A series of DNA samples containing from 2 to 10 000 copies of HIV DNA was prepared by diluting DNA from 8E5 cells with DNA from a seronegative individual. These samples, each containing 1 μg of DNA, were subjected to PCR amplification and subsequent detection by the microplate hybridization assay, the ^{32}P-probe solution hybridization assay, and th AE probe assay. The microplate assay detected DNA quantitatively in the range tested (Figure 2). The enzymatic generation of colored product from the assay of only 5 μL of amplified product was so intense that standards with a pre-PCR HIV content of ≥32 copies gave absorbance readings at 450 nm >1.5 *A*. These PCR products had to be diluted and assayed again in the microplate assay. Interrun variations, examined by three independent assays performed by the same individual, were similar to those for intrarun variations.

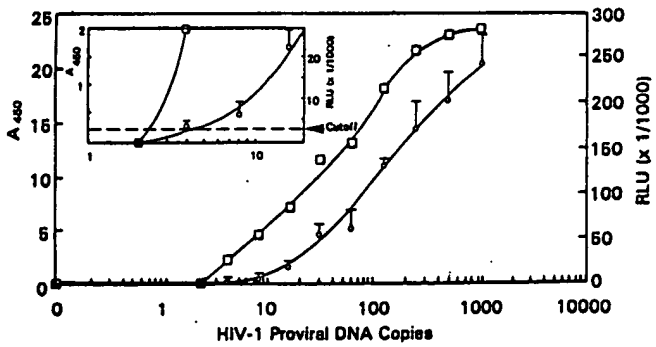

Fig. 2. Detection of HIV-1 proviral DNA

○, 5 μL of amplified sample was assayed by the microplate hybridization assay, A_{450} results; □, 25 μL of amplified sample was assayed by the AE probe assay, results in RLU. For the microplate assay, each point represents an average from three parallel assays; vertical bars are 1 SD. The AE probe was only run once

We compared this colorimetric assay with the ^{32}P-labeled probe and AE probe methods requiring 10 and 25 μL of amplified product, respectively. The AE probe assay was only run once because of the large volume of amplified sample (25 μL) required for the assay. Intrarun variation of the AE probe assay was assessed previously (*6*). As shown in Figure 2, the detection sensitivities (4 HIV proviral c pies per 150 000 cells) of the microplate hybridization and AE probe assays were comparable. We previously showed that the detection limits of the AE probe and solution ^{32}P hybridization assays were comparable (*6*). Here, using a ^{32}P-labeled oligonucleotide probe, SK19 (*2*), we found the same detection limit as the microplate hybridization and AE probe assays (data not shown).

Detection of HIV-1 DNA in PBMC Samples

To investigate the performance of the microplate assay in clinical specimens, we examined 104 seronegative and 142 seropositive individuals. Five and 25 μL of the amplified products were assayed by the microplate assay (Figure 3) and by the AE probe, respectively. All DNA samples from seronegative individuals tested negative by both methods. The RLU readings of the AE probe method ranged from 200 to 5380, a range below the positive cutoff of 10 000 RLU established in our laboratory in the last 2 years (*6*). The colorimetric readings of these samples ranged from 0.011 to 0.265 *A*. The average ± 3SDs (99% confidence level) of the 104 negative specimens was 0.113 ± 0.156 *A*. The mean + 3 SD (0.269 *A*) was used as the cutoff value for seronegativity. All but one amplified DNA sample from the 142 seropositive individuals tested were positive by both methods. The RLU readings of the AE probe method ranged from 12 421 to >400 000 for positive samples. Of the specimens, 84% (119 of 142) gave an A_{450} reading >2.0 in the microplate assay. After a fivefold dilution, 60% of the specimens (62 of 104) still gave readings >2.0 *A* (i.e., the adjusted reading was >10.0 *A*). After a 10-fold dilution, 33% (34 of 104) gave readings of >2.0 *A* (adjusted readings of >20.0 *A*). Thus, the microplate assay provided a clear discrimination between seropositive and seronegative individuals.

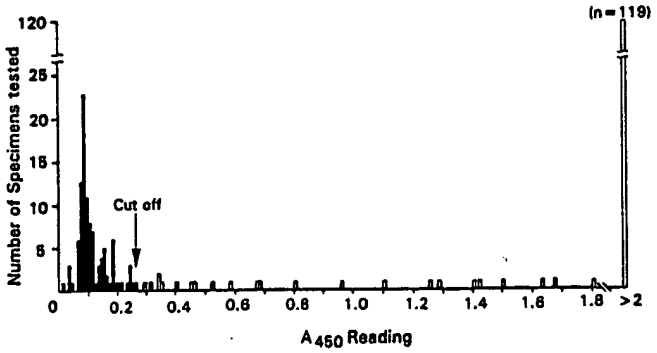

Fig. 3. Specimens derived from seropositiv (n = 142, *open bars*) and seronegativ (n = 104, *dark bars*) individuals

Discussi n

There are several ways to detect amplified HIV product. Direct visual inspection of amplified DNA from clinical specimens by gel electrophoresis and ethidium bromide staining are useful but are often complicated by the presence of other amplified DNA with similar electrophoretic mobility. Analysis of the amplified DNA by electrophoretic separation and Southern blotting and hybridization with a ^{32}P-labeled probe is more sensitive and specific than ethidium bromide staining but requires considerably more laboratory work. Most laboratories use solution hybridization to increase the sensitivity and eliminate the blotting process (*2, 3, 12–14*). After hybridization, positive samples yield a band with a different electrophoretic mobility than that of the unbound probe. Autoradiographic detection requires 3–18 h. Because this method uses ^{32}P-labeled probes, the half-life of the probe and disposal of reagents present a major problem to many diagnostic laboratories.

The AE method is quantitative with a detection limit comparable with that of solution hybridization with ^{32}P-labeled probes (*6*). However, a disadvantage of the AE probe assay is the required use of a luminometer and single tube readings immediately after stopping the reaction. The microplate hybridization assay presented here offers a new approach to measuring proviral DNA in seropositive individuals. It differs from previously reported enzyme immunoassays that include microwells or beads coated with avidin or anti-biotin antibody to bind the hybridization complex (*15–19*). Here, an oligonucleotide DNA probe, the capture probe, bound directly to the well of a microtiter plate or a microwell strip, hybridizes with the target DNA. Subsequent hybridizations of a T-tailed oligodeoxynucleotide (the signal probe) to the target DNA and of biotin-labeled poly dA to the signal probe introduce the biotin label to the hybridization complex. Readings are made with a standard microplate reader, which is present in most clinical laboratories, and wells containing clearly positive samples are easily detected by eye. When high-volume testing is required, the assay can be performed in an entire 96-well microplate; when fewer assays are done, the test can be performed in microwell strips, with use of groups of 8–12 assays per strip. Furthermore, all sample preparation, hybridization, and detection steps are performed at room temperature and, except for DNA sample denaturation, which is performed in a separate tube, all steps are performed on the same plate.

Patients with symptomatic infections were reported to have more HIV in their PBMC and plasma, as determined by endpoint dilution culture, than do patients with asymptomatic infections (*20, 21*). As demonstrated here, the microplate method can be combined with PCR for quantitative assessment of viral copies. Thus, this procedure provides the potential for direct assessment of viral loads in patients' samples. This detection system can also be coupled with a thermocycler formatted for a 96-well microplate to provide greater consistency in both amplification and detection.

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